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REVIEW

The dynamic nature and regulation of the root clock

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ABSTRACT

Plants explore the soil by continuously expanding their root system, a process that depends on the production of lateral roots (LRs). Sites where LRs can be produced are specified in the primary root axis through a pre-patterning mechanism, determined by a biological clock that is coordinated by temporal signals and positional cues. This 'root clock' generates an oscillatory signal that is translated into a developmental cue to specify a set of founder cells for LR formation. In this Review, we summarize recent findings that shed light on the mechanisms underlying the oscillatory signal and discuss how a periodic signal contributes to the conversion of founder cells into LR primordia. We also provide an overview of the phases of the root clock that may be influenced by endogenous factors, such as the plant hormone auxin, and by exogenous environmental cues. Finally, we discuss additional aspects of the root-branching process that act independently of the root clock.

KEY WORDS: Lateral root formation, Root clock, Pre-patterning, Oscillation, Pre-branch site, Auxin, Environmental signals

Introduction

The plant kingdom is characterized by exceptional plasticity in post-embryogenesis organ formation. Throughout their entire lifespan, plants repetitively produce new organs, such as leaves and lateral roots (LRs) above and below ground, respectively. LRs anchor the plant in the soil where they take up water and nutrients. The branching of LRs follows a regularly spaced pattern along the primary root that then transmits underground environmental cues to the aerial organs. LR development represents an excellent model for dissecting the molecular mechanisms underlying organ formation, because it requires the coordination of complex spatial and temporal signals.

Over the last decade, the signal transduction mechanisms regulating the development of LRs have been intensively studied and recently reviewed (Du and Scheres, 2018; Santos Teixeira and ten Tusscher, 2019; Motte et al., 2019). Briefly, a LR originates from a patch of 8-15 xylem pole pericycle cells [XPP; see Glossary, Box 1) (von Wangenheim et al. (2016)], which are situated deep inside the longitudinal axis of the primary root (Dubrovsky et al., 2008). Within this group of XPP cells, a pre-patterning event specifies two central XPP cells to become founder cells. The founder cells undergo nuclear migration and asymmetric cell division to generate a lateral root primordium (LRP; see Glossary, Box 1) (De Rybel et al., 2010; De Smet et al., 2008). The process of lateral organ formation relies on a biological clock that periodically translates a temporal signal into spatial information along the primary root axis (Moreno-Risueno and Benfey, 2011; Van Norman et al., 2013), and thereby determines the time and place for a set of XPP cells to be selected for founder cell specification.

Molecular evidence has shown that the root clock is characterized by the oscillating expression of genes in a region close to the tip of the primary root, called the oscillation zone (OZ; see Glossary, Box 1). The temporal change of gene expression in the OZ is translated into the repetitive formation of pre-branch sites (see Glossary, Box 1), the location of which can be approximated to LR founder cell specification. Subsequently, several rounds of cell divisions of the founder cells and neighboring pericycle cells are combined with cell swelling to establish a dome-shaped LRP. As the LRP grows, it breaks through the overlying endodermal, cortical and, finally, epidermal cells of the primary root to emerge as a LR (Lucas et al., 2013; von Wangenheim et al., 2016). Cell-to-cell communication between the growing LRP and the overlying tissues is required to activate local cell wall 'loosening' of the endodermal and cortical cells to allow the further outgrowth of LR (Vermeer et al., 2014). Therefore, the activity of the root clock results in a regularly-spaced pattern of LRs along the primary root axis, thus acting as the most upstream signal known to trigger LR formation.

Following the exposition of the up-to-date described components of the root clock, we discuss in this Review the currently known signals regulating oscillation periodicity or amplitude (see Glossary, Box 1) that eventually control the establishment of pre-branch sites. Second, we examine the signals capable of activating the translation of a pre-branch site into a LRP, which in turn controls lateral root spacing, and we discuss how auxin signaling and transport can be involved in modulating the oscillations. In addition, we provide an overview of potential external factors that may influence the root clock. Finally, we refer to a part of the root-branching process that potentially acts independently of the root clock in plants, and the factors that might affect this process.

Spatiotemporal regulation of the root clock

The root clock can be visualized by the use of the *DR5* promoter (see Glossary, Box 1) fused to a luciferase reporter (*DR5:Luciferase*) (Moreno-Risueno et al., 2010). The use of a luciferase reporter enables the detection of the dynamics of the auxin output response, because luciferase has a half-life of about 3-6 h (Moreno-Risueno et al., 2010). Time-lapse imaging of the *DR5:Luciferase* signal in the root reveals an oscillatory expression pattern in the OZ (Fig. 1), which might be explained by a negative-feedback model (Fig. 2). In this model, we hypothesize that local auxin signaling induces the degradation of small repressor proteins, known as AUX/IAA proteins (see Glossary, Box 1), which inhibit auxin response factors (ARFs). The degradation of the AUX/IAA proteins alleviates the inhibition of ARFs that then activate the AuxREs present in the *DR5* promoter. In parallel, the local auxin accumulation also steers the transcription of auxin-inducible genes, including AUX/IAA-coding genes, replenishing the AUX/

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Box 1. Glossary

AUX/IAA proteins. Auxin/INDOLE-3-ACETIC ACID (AUX/IAA) proteins are transcriptional repressors that are core components of the TRANSPORT INHIBITOR RESPONSE/AUXIN F-BOX (TIR1/AFB)-AUX/IAA co-receptor system for auxin perception (Lavy and Estelle, 2016; Salehin et al., 2015). AUX/IAA proteins comprise 29 family members, and are short-lived proteins that are rapidly degraded by SKP1 CULLIN F-BOXTIR1/AFB (SCFTIR1/AFB) ubiquitin protein ligases (E3s) in the presence of auxin. Each AUX/IAA protein has been suggested to interact, and to negatively regulate, the activity of one or more auxin response factors (ARFs). The degradation of AUX/IAA proteins releases the inhibition of ARF activity, resulting in the trans-activation of auxin-responsive gene transcription. So far, several AUX/IAA proteins (e.g. IAA3, IAA14, IAA28 and IAA19) have been identified to regulate lateral root development during different stages (Péret et al., 2009).

Auxin reflux model. Directional auxin movement created by the arrangement of both auxin influx and efflux facilitator proteins in the root apex. While the auxin influx carriers are responsible for auxin uptake, the polar-localized PIN proteins in the plasma membrane determine the directionality of the auxin flux. Auxin is first observed to accumulate in the quiescent center and root cap surrounding cells (i.e. columella), as a result of the rootward auxin transport and local auxin biosynthesis. Then, it is taken up by neighboring lateral root cap cells and further transported through the epidermis in a shootward direction, and finally reaches the elongation zone to trigger a local maximum auxin response.

DR5. *DR5* (*DIRECT REPEAT5*) is a highly active and widespread reporter of auxin transcriptional response, which consists of 7-9 auxin response elements (AuxREs) containing tandem direct repeats of 11 base pairs that include the auxin-responsive TGTCTC element found in the soybean *GH3* promoter (Ulmasov et al., 1997). These elements are crucial in the control on gene expression downstream of the TIR1/AFB-AUX/IAA-ARF signaling module. By fusing the *DR5* promoter or its reverse complemented sequence (*DR5rev*) to different reporter genes, e.g. Luciferase, GUS, GFP and VENUS, the auxin response has been found to occur specifically in the tissues that are correlated with lateral root development. Several *DR5* reporter versions have been developed. Typically, Luciferase and GUS are transcriptionally fused under the control of the promoter described by Ulmasov et al. (1997). Those constructs are herein below mentioned as *DR5:Luciferase* and *DR5:GUS*, respectively. Another version, *DR5rev*, has been fused to endoplasmic reticulum-tagged GFP (Friml et al., 2003) and 3xVENUS-N7 (Heisler et al., 2005).

Lateral root cap (LRC). The primary root tip is surrounded by extra layers of cells called 'root cap' that protect the root apical meristem. Distinction is made between two groups of cells: first, the ones in the close vicinity of the quiescent center and the tissue initial cells at the very tip of the root are defined as the 'columella'. Second, the ones prolonging the columella in a shootward direction and enrobing the root epidermis up to the meristematic zone end are referred as the 'lateral root cap (LRC)'. The LRC plays a role in lateral root formation.

Lateral root cap programmed cell death (LRC-PCD). At the most distal end of the lateral root cap, a group of cells perceive a programmed cell death signal and undergo cell death by nuclear lysis within a short time period. This PCD signal is periodically activated and results in the translocation of auxin from the dying LRC cells to neighboring epidermal cells through lateral root cap- and epidermis-localized auxin influx and efflux carriers.

Lateral root primordium (LRP). The first visible step of lateral root development is the migration of the nuclei of neighboring founder cells towards their common cell walls followed by anticlinal asymmetric cell divisions. After this first round of cell divisions, smaller cells flanked by larger daughter cells are produced, giving rise to a first recognizable stage of lateral root development, 'stage I'. The event of producing such a stage is defined as 'lateral root initiation'. Through subsequent cell divisions, now also including periclinal divisions, a layered structure is formed inside the parent root, which is referred to as lateral root primordium. The number of cell layers are used to determine the development stage (stages II to VIII). The LRP finally emerges from the parent root after breaking its epidermis and is then defined as a lateral root.

Oscillation amplitude. Oscillation amplitude is the change between the highest and the lowest expression level in the expression profile over time for a given oscillating gene.

Oscillation periodicity. The time between consecutive cycles of gene expression that occur in the oscillation zone.

Oscillation zone (OZ). A region of the primary root approximately situated in the elongation zone and characterized by an oscillating expression of the *DR5:Luciferase* reporter. Expression of some endogenous genes oscillate in phase and other in anti-phase with *DR5:Luciferase* expression.

Pre-branch sites. Pre-branch sites are defined as steady spots of *DR5:Luciferase* signal in the differentiation zone following an oscillation peak (maximum amplitude). Because of the lack of cellular resolution in *DR5:Luciferase* live-imaging systems, it is currently impossible to identify the developmental steps ongoing at a pre-branch site. A pre-branch site can therefore be a founder cell, an initiating lateral root and/or any later stages of primordia development. Nonetheless, as those developmental events take place in a sequential order, pre-branch site formation can be assimilated to founder cell specification.

Xylem pole pericycle cells (XPP cells). In the model species *Arabidopsis*, lateral roots grow out radially from the primary root and originate from a tissue named 'pericycle'. The pericycle is the outermost layer of the vascular bundle and is situated within the three outermost concentric tissues, i.e. (from outside to inside) epidermis, cortex and endodermis. The *Arabidopsis* root has two protoxylem poles oriented to the outside and joined at the center by the formation of metaxylem. The pericycle cells that are positioned next to the protoxylem poles are referred to as xylem pole pericycle cells. In *Arabidopsis*, lateral roots develop only from these cells. Nonetheless, only subsets of XPP cells, regularly spaced along the primary root, grow into lateral roots. For this reason, they are named 'founder cells'.

IAA pools. The resulting accumulation of AUX/IAA repressive factors then inhibit ARF-dependent transcription and *DR5:Luciferase* expression. Therefore, oscillations of the *DR5* signal in the OZ can occur in a cyclic model, which is synchronized with the activation/inactivation of ARFs, coupled with the stabilization/degradation of AUX/IAA proteins by a local auxin input (Kircher and Schopfer, 2018).

The transient accumulation of *DR5:Luciferase* in the OZ suggests that the cells undergo a temporary auxin response when passing through the OZ (Moreno-Risueno et al., 2010). Although it does not immediately result in the induction of a new LRP, reaching a maximum auxin response in the OZ is required to later induce lateral organ formation (Xuan et al., 2015). Without a clear insight into the molecular mechanisms that occur in the OZ, auxin signaling is tentatively proposed to prepare XPP cells for LR formation.

Unfortunately, it is difficult to precisely identify the cell types and tissues that are affected by the oscillation, owing to the lack of sufficient cellular resolution when imaging the luciferase marker (Van Norman et al., 2013). Such constraint has been circumvented by using the GUS reporter system, where β-glucuronidase activity can be visualized at the cellular level after transformation of its substrate (X-Gluc) into a blue-colored precipitate. *DR5:GUS* expression has been observed at regular time intervals in the transition zone between meristematic and elongation zones (i.e. the basal meristem) and correlates with the periodicity of LR formation (De Smet et al., 2007). Moreover, microscopic analysis has revealed that *DR5:GUS* is specifically expressed in the basal meristem protoxylem strands (i.e. the xylem pole strands; see XPP in Glossary, Box 1). Although the involvement of other cell types cannot be excluded, these data suggest that the oscillation signal along the longitudinal axis might preferentially affect protoxylem

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Box 2. Model of *DR5* oscillation frequency

One model predicts that cells of alternating sizes regularly enter the OZ and cause the auxin response oscillations observed in this region of the root (van den Berg and ten Tusscher, 2018 preprint). More specifically, during root growth, individual cells arise sequentially as progeny from the stem cells in the very tip of the primary root, neighboring the QC. These daughter cells proliferate, thereby creating clones of sibling cells with synchronized growth and division rates. Depending on the size of the cell at the moment it enters the OZ, it will accumulate different levels of auxin: larger cells take up more auxin, because auxin import increases with membrane surface area. Meanwhile auxin efflux remains unchanged because the auxin efflux carriers are localized to the poles of the non-expanding basal membranes of the elongating cells. Thus, according to this model, the first cells that enter the OZ will be smaller than their more rootwardly situated sister cells, have a relatively low level of auxin uptake capacity and cannot induce a measurable auxin response. However, when the last cells from the clone arrive in the OZ, their size is considerably larger and contains enough auxin to induce the auxin-response machinery. Assuming such a decisive role of cell division dynamics for the generation of the oscillations, the faster cell division meristems of 8-day-old seedlings, when compared with the slower growth of just germinated seedlings, might explain the huge differences in oscillation frequencies. In faster growing roots, more cells per time unit will enter the OZ, correlated with a faster sequence of small and larger cells.

cells exiting the basal meristem and entering the OZ. Consequently, an auxin-induced signal derived from protoxylem cells might be responsible for specifying the neighboring pericycle cells that develop into a LR (De Smet et al., 2007). This process has been named 'priming', but what this represents at the molecular level still needs further investigation. Following an oscillation maximum (see Glossary, Box 1, pre-branch site) (Fig. 2), the auxin response decreases for a short time, before it increases again to a point that a stable *DR5:Luciferase* signal can be detected. This signal corresponds to the formation of a pre-branch site, from which a LR can develop. Remarkably, a pre-branch site is created in a broad developmental window where, overall, the auxin response in the root is minimal (Dubrovsky et al., 2011). Within this region of minimal auxin response, the input of auxin is restricted to a few cells and a founder cell specification reporter gene, *GATA23* (a member of the GATA transcription factor family), becomes expressed specifically in XPP cells (De Rybel et al., 2010). However, its expression is not confined to a few cells but rather becomes expressed in a long (and not-well defined) stretch of XPP cells in the OZ. Thus, above the OZ, *pGATA23:NLS-GFP* becomes restricted to a few XPP cells, overlapping with the *DR5* expression in the newly specified pre-branch site. These findings indicate that the oscillation generates local signal transduction to specify a set of XPP cells for founder cell establishment, by which the temporal input of auxin signal is translated into a continued developmental signal for LR initiation (LRI).

Timing of the root clock

The oscillations result in regular spacing of pre-branch sites, and consequently LRs, along the primary root. However, quantification of the oscillation periodicity at different seedling stages after germination has revealed variation in the timing of the root clock, ranging from an interval of ∼4-15 h between two pre-branch sites (De Smet et al., 2007; Kircher and Schopfer, 2016; Moreno-Risueno et al., 2010; Xuan et al., 2015). The lowest periodicity of pre-branch site formation (i.e. 15 h) is observed during the first day after germination (De Smet et al., 2007), whereas the frequency increases to a periodicity of 6 h in 2- to 3-day-old seedlings and to 4 h in 4-day-old seedlings (Kircher and Schopfer, 2016; Xuan et al., 2015). This might be caused by the different growth conditions applied in these experiments (e.g. light intensity and light/dark period, and nutrient supply); however, more recently a detailed quantification of the production rate of pre-branch sites over a period of 8 days starting from germination was performed under steady growth conditions (i.e. continuous light and sufficient nutrient supply) and confirmed the influence of seedling age on the periodicity of the root clock (Xuan et al., 2015). This study revealed a gradual increase in the periodicity of pre-branch site formation during the early development stages of the seedlings, reaching a stable rate of 2 h per cycle in 8-day-old seedlings (Xuan et al., 2015). The increased production rate of pre-branch sites shares a similar trend with the increasing meristem size, which is gradually expanding following germination, owing to an enhanced cell division rate that surpasses the rate of cell differentiation during the early phase of the seedling growth. Interestingly, a computational approach recently proposed that the *DR5* oscillation frequency could indeed be dependent on meristematic cell cycle duration (Box 2) (van den Berg and ten Tusscher, 2018 preprint).

In addition, differential cell elongation is central to the gravitropic bending of roots and, interestingly, the timing of the root clock can be shortened by gravistimulation. A strong change in the primary root tip growth direction is induced by altering the orientation of the root by ≥90°, and the time interval between consecutive *DR5:Luciferase* oscillations is reduced to half that required under normal conditions (Moreno-Risueno et al., 2010; Xuan et al., 2016). However, manually bending the root at the differentiation zone to form a J-hook (root curve turned in a J shape, Laskowski et al., 2008) failed to induce extra pre-branch sites at the bending site (Moreno-Risueno et al., 2010), indicating that the changes of the periodicity involve a signal localized in the primary root tip. In the case of gravistimulation, the redirection of the root tip accelerates auxin flux in the lateral root cap (LRC, see Glossary, Box 1) and in the neighboring epidermis cells in the elongation zone via auxin transporters (e.g. AUX1), leading to a transient auxin accumulation in the elongation zone (Band et al., 2012; Swarup et al., 2005). Meanwhile, AUX1 also mediates shootward auxin transport throughout the LRC and epidermis to regulate root gravitropism and the formation of pre-branch sites and LRs (De Smet et al., 2007; Xuan et al., 2016). Therefore, the fluctuation of endogenous auxin in OZ might account for the changed timing of the oscillation under gravitropic bending. Finally, calcium signaling might also be involved in the impact of gravity on the timing of the root clock. Upon gravity stimulation, cytosolic calcium transiently increases in the pericycle cells, and blocking this calcium signal represses the bending-induced LR formation (Richter et al., 2009).

Factors triggering the oscillation signals

In the OZ, the oscillation acts as the upstream signal that triggers the establishment of pre-branch sites for LRs. Indeed, interfering with the oscillations alters pre-branch site formation (Moreno-Risueno et al., 2010; Van Norman et al., 2014; Xuan et al., 2016). So far, several signaling cascades that are mediated by transcription factors, auxin and mechanical signals are known to be required for maintaining the formal activation of this temporal signal (discussed below).

Transcriptional control by oscillating genes

Transcriptome analyses of dynamic gene expression in the OZ showed that a broad range of genes are oscillating either in-phase (2084 genes) or anti-phase (1409 genes) with *DR5:Luciferase* expression (Moreno-Risueno et al., 2010). Among them, several MADS-box protein family transcription factors, such as SHATTERPROOF1

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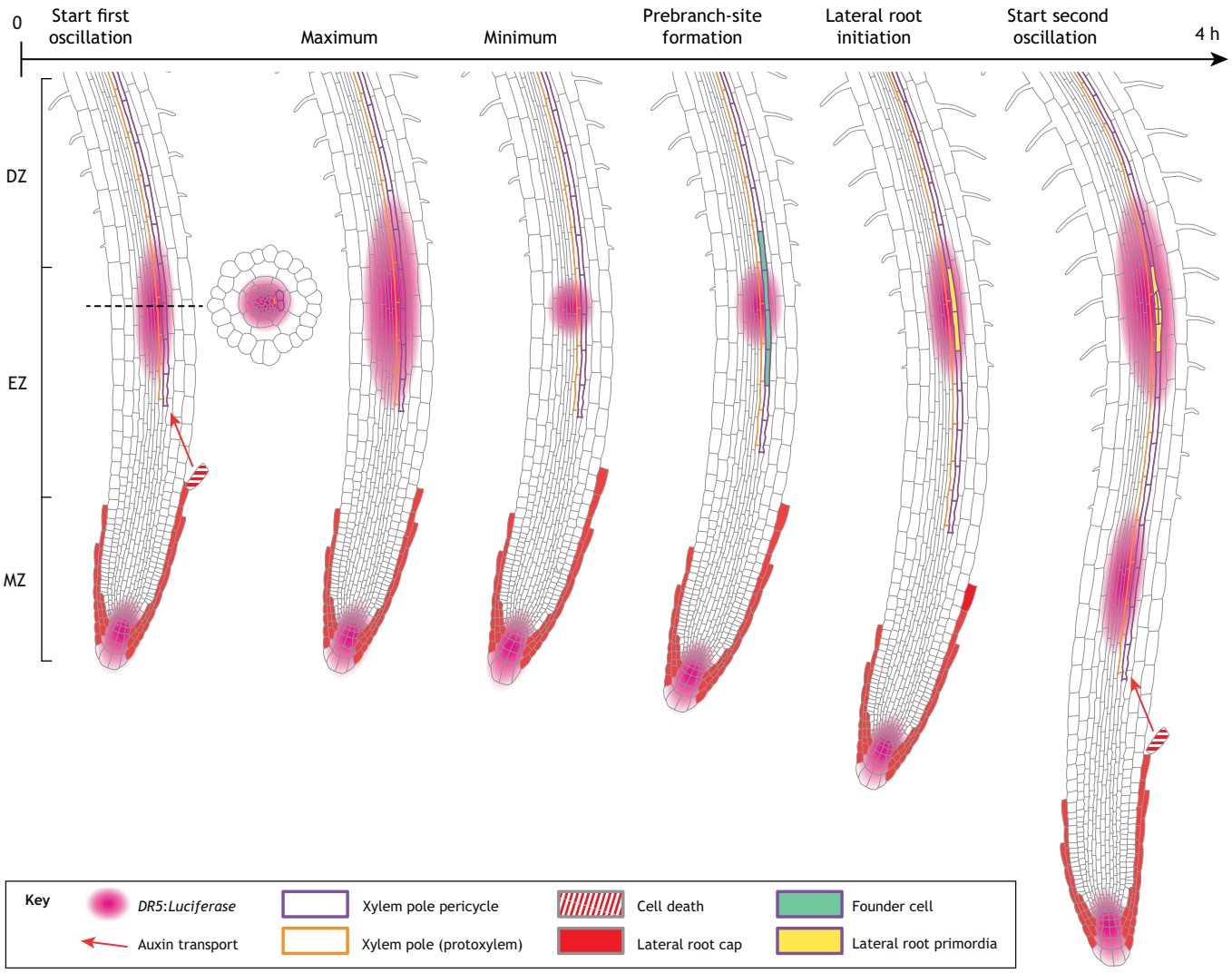

Fig. 1. Scheme showing a time unit (4 h) of the root clock. It is hypothesized that the clock is set by a periodic signal triggering programmed cell death (PCD) in the most distal lateral root cap (LRC) cells (red). Deposited auxin from dying LRC cells (red stripes) is transported into the inner tissue of the root (red arrow), promoting the formation of an auxin response maximum, as visualized using the *DR5:Luciferase* reporter (pink), at the distal part of the elongation zone. Subsequently, the auxin maximum is temporarily attenuated before accruing gradually again, which coincides with the accumulation of auxin response in xylem pole pericycle (XPP) cells (purple border). The latter has been interpreted as crucial for specifying the XPP cells as founder cells (green), which later undergo lateral root initiation to become a lateral root primordium (yellow). This process is repeated when the next LRC cells perceive the cyclic PCD signal and undergo cell death. DZ, differentiation zone; EZ, elongation zone; MZ, meristematic zone.

(SHP1), SHATTERPROOF2 (SHP2) and SEEDSTICK (STK), have been identified to show an oscillating gene expression in the OZ in phase with *DR5*. These genes might regulate the periodicity of the root clock, as *shp1shp2* and *shp1shp2stk* mutants both display arrhythmic periodicity of the *DR5* oscillation and a decreased number of pre-branch sites (Moreno-Risueno et al., 2010). The NAC-domain transcriptional factor genes *VND2*, *FEZ* and *SOMBRERO* (*SMB*), which exhibit oscillating gene expressions in phase with *DR5*, are also involved in regulating the root clock. Mutations in these genes change oscillation frequency and reduce the number of pre-branch sites (Moreno-Risueno et al., 2010). Unexpectedly, only a few known LRI genes or auxin-related genes are represented in this OZ dataset, implying that oscillation activity and LRI might be differentially regulated at the transcriptional level. Among the genes that are represented, auxin response factor 7 (ARF7), which shows an anti-phase expression profile compared with DR5 in the OZ, has been proposed to contribute to oscillation periodicity. ARF7 is also involved in IAA28-dependent founder cell specification, which happens shortly after oscillation, and might function in connecting oscillation with founder cell specification.

Cyclic programmed cell death in the lateral root cap

Remarkably, the oscillating gene *SMB* is a key regulator of LRC cell maturation and programmed cell death (LRC-PCD, see Glossary, Box 1) (Bennett et al., 2010; Fendrych et al., 2014; Willemsen et al., 2008). It is specifically expressed in root cap cells but is absent from the OZ, suggesting non-cell autonomous action of SMB on the root clock. The LRC typically terminates where the meristematic cells stop dividing and start to elongate. At the distal end of the LRC, clusters of maturated root cap cells perceive a cyclic PCD signal and are shed from the root tip with a periodicity equal to that of the *DR5* oscillation in the OZ (Xuan et al., 2016). PCD of the LRC cells precedes the oscillation signal in the OZ, and therefore has previously been interpreted to regulate the oscillations. In the

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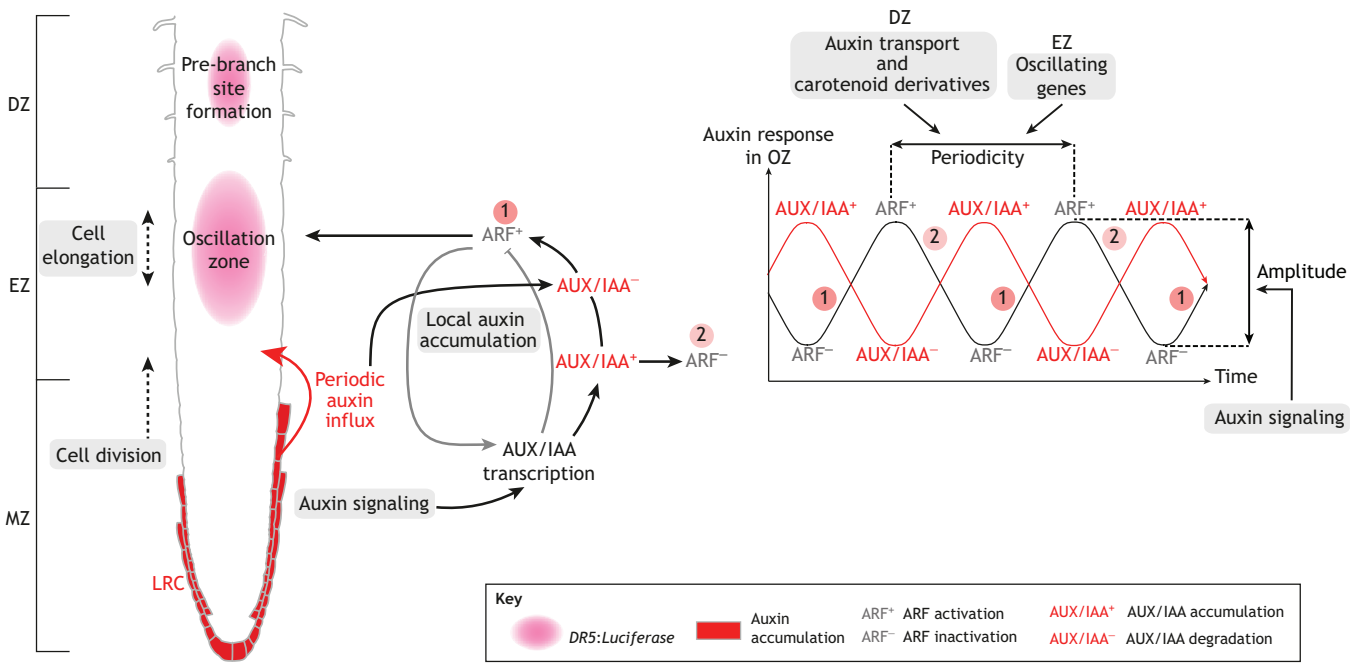

Fig. 2. The putative molecular mechanisms underlying the fluctuation of *DR5:Luciferase* expression in the elongation zone of the root. In the meristematic zone, the lateral root cap serves as a source of auxin, which is transported to the main root (red arrow). Auxin accumulates in the elongation zone, resulting in the local degradation of AUX/IAA proteins (1). This, in turn, leads to the activation of AUXIN RESPONSE FACTORs (ARFs), which triggers an auxin responsive *DR5* expression peak (pink). The activation of ARFs will, however, also induce *AUX/IAA* transcription and AUX/IAA proteins will start to accumulate again (2), thus repressing the ARF activity and *DR5* expression, in a negative-feedback loop. This process occurs at regular time intervals (i.e. oscillation frequency) under the control of the oscillating genes, while the *DR5* signal intensity (i.e. oscillation amplitude) is maintained by local auxin accumulation and signaling. In the elongation zone, the fluctuation of *DR5* expression is supposedly determined by the cyclic activation and inactivation of ARFs directed by AUX/IAA protein turnover. Finally, *DR5* oscillation is also facilitated by auxin transport and so far uncharacterized carotenoid derivatives derived from more shootwardly situated tissues. DZ, differentiation zone; EZ, elongation zone; MZ, meristematic zone.

conditions where LRC-PCD is delayed, the frequency of pre-branch site formation is decreased accordingly. Conversely, accelerated PCD in the LRC completely interrupts the *DR5* oscillation pattern and stops pre-branch site formation, as shown by overexpressing SMB in an inducible manner (Xuan et al., 2016). Rescuing the root from SMB overexpression restores root cap formation, LRC-PCD and pre-branch site formation. Therefore, the PCD signal in LRC might act as the temporal signal that regulates root cap dynamics, which in turn functions as a mechanical cue for the root clock. However, it has not yet been determined how the oscillating genes identified in the above-mentioned transcriptome analyses respond to the PCD signal and root cap dynamics.

Auxin transport

In *Arabidopsis*, the establishment of a local auxin gradient through auxin flux carriers and diffusion is required for organ formation (Benková et al., 2003; Grieneisen et al., 2007). Auxin is also involved in regulating the oscillations of *DR5* expression (Laskowski and ten Tusscher, 2017). The root cap is an important source for auxin and sustains high auxin levels, especially in the cells at the most distal end of the LRC that will finally undergo PCD. A computational approach has revealed that LRC-PCD contributes to the root clock by redistributing auxin from the dying root cap cells back into the primary root (Xuan et al., 2016). In this model, LRC-specific auxin biosynthesis, as well as symplastic and apoplastic auxin transport are integrated. Upon simulation of the LRC-PCD, a high level of auxin is converted from indole-3-butyric acid (IBA) in the dying LRC. This auxin is predicted to be taken up by the neighboring epidermal cells and transported to the inside of the primary root to cause an auxin response in the OZ, followed by specification of XPP cells for LR formation. This process is likely coordinated by both rootward and shootward auxin transport, and is mediated by tissue-specific expression of multiple auxin influx carriers and efflux carriers (e.g. PIN2, ABC transporters ABCB1/19) – essential components of the ‘auxin reflux model’ (see Glossary, Box 1) (Blilou et al., 2005). The AUX1 influx protein localizes to the plasma membranes of LRC cells and to outer membranes of epidermal cells, therefore maintaining high auxin levels in the LRC and facilitating auxin uptake by the primary root (Fig. 3). The *aux1* mutant has reduced auxin levels in the LRC and epidermal cells, which inhibits *DR5* oscillation and pre-branch site formation. The localization of PIN2 and ABCB1/19 overlaps in the LRC, epidermis and cortex, to ensure the transport of auxin from the LRC into the central tissues of the primary root (Xuan et al., 2016). Remarkably, PIN2 exhibits a specific polar localization on the inner side of the dying LRC cells facing the epidermal cells, indicative of the possible direction of the auxin stream from the LRC to the epidermis (Xuan et al., 2016) (Fig. 3). However, PIN2 and ABCB1/19 are functionally redundant in regulating LR formation, because mutations in the encoding genes only slightly reduce LR formation (Xuan et al., 2016). In contrast, the treatments with either the chemical auxin efflux inhibitors NPA (N-1-naphthylphthalamic acid) or BUM (2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid) both completely abolish DR5 oscillation and pre-branch site formation by putatively blocking the inward auxin transport from the root cap to the tissues of the root central cylinder, thereby retaining the auxin in the outer layer tissues. Thus, PIN- and/or ABCB-mediated polar auxin transport is clearly required for this process but other – yet to be identified – auxin efflux carriers might be involved as well.

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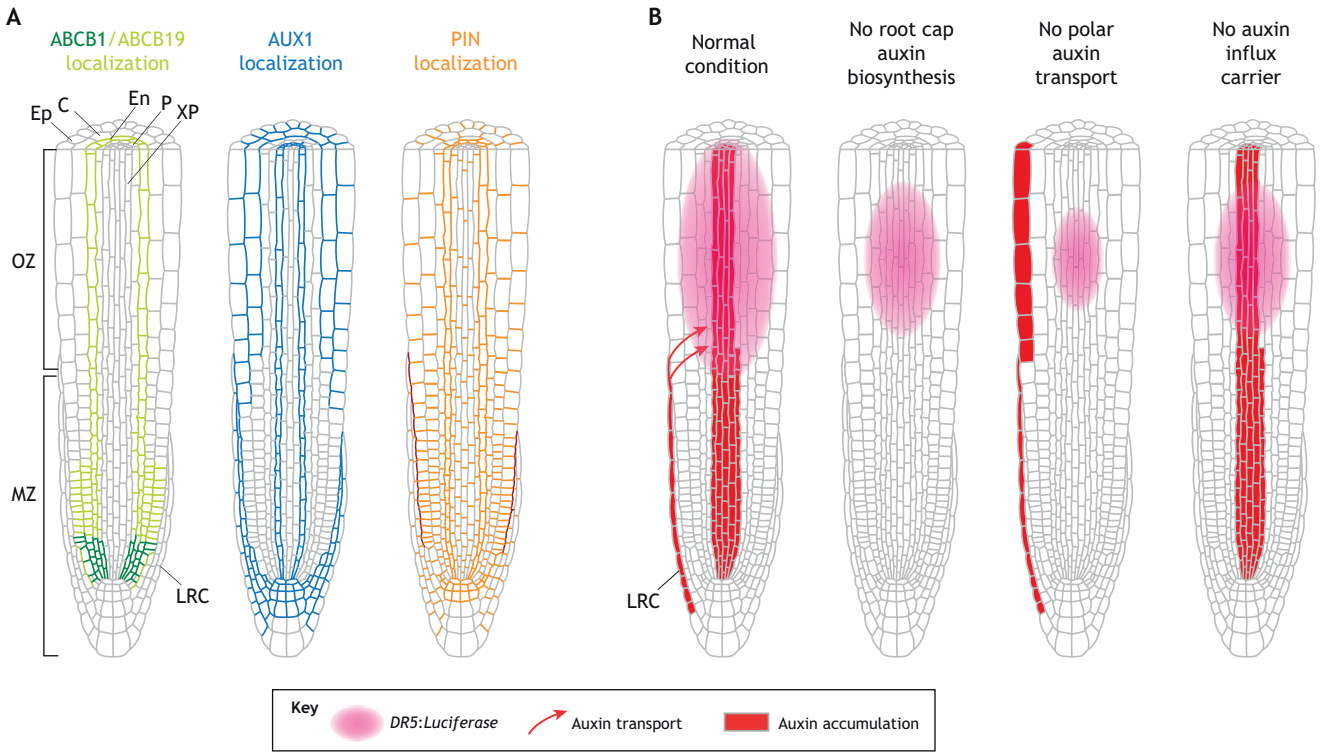

Fig. 3. Contribution of auxin transport to the oscillations. (A) Schematic representations of the *Arabidopsis* root tip indicating the distribution of auxin influx and efflux carriers in the root tip. Colors indicate the tissue-specific localization of these auxin transport proteins. The inner side of the lateral root cap (LRC) cells facing the epidermal cells in the PIN localization overview are darker red. This represents the specific PIN2 localization indicative of the possible direction of the auxin stream from the LRC to the epidermis. (B) Altered auxin distribution under different auxin defective conditions. Under normal conditions, auxin accumulates at high levels in LRC and vascular tissues, while the lack of root cap-derived auxin biosynthesis leads to a minimal low level of auxin in these tissues. In the case of auxin transport, disruption of polar auxin transport results in reduced auxin in the vascular tissue but in an accumulation of auxin in the LRC and epidermis; blocking the auxin influx carrier-mediated auxin transport strongly reduces auxin levels in the LRC. C, cortex; En, endodermis; Ep, epidermis; MZ, meristematic zone; OZ, oscillation zone; P, pericycle; XP, xylem pole (protoxylem).

Carotenoid derivatives

An uncharacterized carotenoid-derived molecule is required for maintaining the oscillation signal, because the inhibition of either carotenoid synthesis with CPTA (2-[4-chlorophenylthio]-triethylamine hydrochloride) or the carotenoid cleavage pathway with an aryl-C3N hydroxamic acid analog D15 causes an aberrant oscillation periodicity with a reduced number of pre-branch sites and LRs (Van Norman et al., 2014). However, a key carotenoid biosynthesis gene, *PHYTOENE SYNTHASE* (*PSY*), is consistently expressed in the differentiation zone and is absent from the OZ, which suggests non-cell autonomous regulation of the root clock by carotenoid-derived molecules. Apocarotenoids are derived from carotenoids by oxidative cleavage and are mobile in plants (Beltran and Stange, 2016), which supports the idea that they may act as a mobile signals to regulate the oscillations. Recently, β-cyclocitral, an endogenous apocarotenoid, has been shown to restore LR formation in the presence of D15, an inhibitor of carotenoid cleavage dioxygenases (Dickinson et al., 2019). However, the promotion of LR formation by β-cyclocitral is attributed to its effect on cell division, because it does not impact on *DR5* oscillation nor LRI. In addition, other known apocarotenoids in plants, such as abscisic acid and strigolactones, fail to restore oscillation upon D15 treatment, indicating that uncharacterized apocarotenoids might be required for activating oscillations. Although strigolactones negatively regulate pre-branch site formation by interacting with the cytokinin-signaling cascade (Jiang et al., 2016), the precise mode of action of strigolactones in the root clock remains unclear.

Translation of oscillation to pre-branch site formation

Pre-branch site formation is not only determined by the periodicity of the oscillation signal but also depends on the amplitude of its signal, which sets a threshold. While the oscillation periodicity is controlled by a temporal signal through the activation of oscillating transcription factors, the oscillation amplitude (see Glossary, Box 1) may be influenced by auxin through its biosynthesis and signaling pathways.

Auxin homeostasis and tissue-specific auxin biosynthesis

The amplitude of auxin response in the OZ positively correlates with local auxin accumulation. Application of the natural auxin IAA to the root tip or specifically in the OZ increases the signaling amplitude of *DR5:Luciferase* in the OZ in a dose-dependent manner (Moreno-Risueno et al., 2010). However, the accumulation of auxin in OZ only occasionally misplaces the pre-branch site without creating extra pre-branch sites. On the other hand, overexpression of an auxin-conjugating gene *GH3.3*, which reduces the endogenous auxin levels, attenuates the *DR5:Luciferase* signal in OZ, resulting in fewer pre-branch sites (Xuan et al., 2015). Thus, a minimum auxin level should be reached in OZ to guarantee the formation of a pre-branch site.

In *Arabidopsis*, auxin is mainly produced by the tryptophan-dependent pathway. Inhibition of this major auxin biosynthesis pathway by kynurenine (KYN) slightly inhibits primary root elongation, but severely represses the oscillation amplitude and therefore pre-branch site formation, indicative of a specific regulatory role of the tryptophan pathway (Kircher and Schopfer, 2018).

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Root cap-specific auxin, derived from its precursor IBA, also regulates the oscillation amplitude. Similar to IAA, either application of IBA or enhancing IBA-to-IAA conversion both increase auxin response in the OZ, and thus promote pre-branch site formation (De Rybel et al., 2012; Xuan et al., 2015). In *ibr1ibr3ibr10* triple and *ech2ibr1ibr3ibr10* quadruple mutants, in which IBA-to-IAA conversion is defective (Strader et al., 2011), the oscillation amplitude and consequently the pre-branch site formation are severely reduced, although the oscillation frequency is unchanged (Xuan et al., 2015). This IBA-to-IAA conversion occurs mainly in the LRC cells, and the converted auxin is subsequently transported into the OZ to induce the local auxin response. Unlike the general production of auxin, this tissue-specific auxin biosynthesis appears to be more effective in promoting pre-branch site formation and acts on the oscillation amplitude in particular (Xuan et al., 2015).

TIR1-dependent auxin signaling

Auxin is detected in the nucleus by its receptor SCF^{TIR1} and F-box proteins (Wang and Estelle, 2014). When auxin signal transduction is blocked (either by the TIR1 inhibitors PEO-IAA and auxinole or in the *tir1afb2* double mutant), *DR5:Luciferase* and *DR5:GUS* signals in the OZ are undetectable, leading to the substantial reduction of pre-branch sites (De Rybel et al., 2010; Kircher and Schopfer, 2018; Xuan et al., 2015). Conversely, *tir1afb2* double mutants do not alter the oscillation frequency (Xuan et al., 2015), suggesting that TIR1-dependent auxin signaling also contributes to the oscillation amplitude.

TIR1-dependent signaling requires the degradation of AUX/IAA transcriptional repressors, and the stabilized forms of AUX/IAA proteins repress LRI in *Arabidopsis*. It has been suggested that IAA encoding genes do not oscillate in the OZ and do not account for the oscillation periodicity (Moreno-Risueno et al., 2010). Indeed, founder cell specification is unaltered in the gain-of-function mutants of *IAA3*, *IAA7*, *IAA12*, *IAA14*, *IAA17* and *IAA19* (De Rybel et al., 2010). However, these mutants do display LR defects (e.g. no LRI in *iaa14*/*slr*, reduced LRI in *iaa19*/*msg2* mutant, and delayed LRP emergence in *iaa3*/*shy2-2* and *iaa12*/*bdl* mutants) (De Smet et al., 2010; Fukaki et al., 2002; Tatematsu et al., 2004; Tian and Reed, 1999), confirming the role of IAA proteins in later stages of LR development. However, auxin response is profoundly reduced in the OZ of the gain-of-function mutant of *IAA28*, one of the AUX/IAA genes with strong expression in the OZ, and leads to a defect in founder cell specification and LRP formation (De Rybel et al., 2010). Thus, IAA28 might act as a downstream component of SCF^{TIR1} to regulate oscillation amplitude.

Lateral root primordium development

As mentioned previously, *DR5* oscillation triggers the formation of pre-branch sites, which contain a patch of cells expressing static (non-oscillatory) *DR5:Luciferase* (Moreno-Risueno et al., 2010). A set of 8-15 XPP cells in the pre-branch site are further specified as founder cells by auxin and undergo asymmetric cell division, which can be visualized by following the dynamic expression of *DR5* fused to fluorescent reporters (De Rybel et al., 2010; Dubrovsky et al., 2008). However, not all pre-branch sites form LRPs, owing to the repression of local auxin signaling or to the inactivation of cell cycle progression.

Lateral inhibition mechanisms ensure a single lateral root primordium develops within one pre-branch site

A pre-branch site normally contains one group of paired founder cells, but in some cases two groups of founder cells vertically adjacent to each other in the longitudinal axis of the primary root have been reported (Toyokura et al., 2019). Nonetheless, only one pair of founder cells undergoes cell division to form a LRP. This process might be regulated by a lateral inhibition mechanism mediated by the auxin antagonist cytokinin, as evidenced by the high frequency of paired LRs in both cytokinin synthesis and signaling mutants (Bielach et al., 2012). Cytokinin is synthesized in founder cells and its neighboring pericycle cells. When two groups of founder cell are specified adjacently, cytokinin is capable to act locally as 'paracrine signal' to repress the initiation of neighboring founder cells, by which only one pair of founder cells becomes selected for further development (Chang et al., 2015; Laplaze et al., 2007).

Recently, a novel peptide-signaling cascade TOSL2-RLK7-PUCHI has been shown to play a crucial role in the selection of founder cells (Toyokura et al., 2019). The TOSL2 peptide is transcriptionally induced by lateral organ boundaries domain 16 (LBD16), in an ARF7- and ARF19-dependent manner. TOSL2 acts downstream of auxin to negatively regulate founder cell specification. The mutants of its receptor RLK7 and downstream target gene *PUCHI* display increased specification frequency of nearby founder cells along the longitudinal axis reflected by a stabilized *DR5:Luciferase* expression in the paired pre-branch sites, eventually leading to a higher number of clustered LRPs. Additionally, multiple very long chain fatty acid (VLCFA) biosynthesis pathway genes, the mutants of which display a high frequency of clustered LRPs that are reminiscent of the *puchi* mutant, are transcriptionally regulated by *PUCHI* (Trinh et al., 2019) and might thus also be involved in the pathway downstream of the TOSL2-RLK7-PUCHI signaling cascade.

It seems that clustered LRPs/LRs are more likely to be produced when the lateral inhibition is not active. A higher frequency of clustered LRPs has been observed in a wide range of mutants, such as mutants of *ARABIDOPSIS CRINKLY4* (*ACR4*) (De Smet et al., 2008), *PLETHORA* (*PLT*) transcription factors (*plt3plt7* and *plt3plt5plt7*) (Hofhuis et al., 2013) and auxin efflux carriers (*pin2pin3pin7*) (Laskowski et al., 2008), as well as overexpression lines or mutants of peptide-encoding genes *CEP5* and *GLV6* (Fernandez et al., 2015; Roberts et al., 2016). It remains to be shown whether the disruption of the regular spaced LR pattern in these lines results from the inactivation of the lateral inhibition process, or through the alteration of oscillation frequency in these mutants.

Factors promoting lateral root primordia development at pre-branch sites

After acquiring founder cell identity, pre-branch sites harboring static *DR5* expression might also be retained as founder cells instead of undergoing asymmetric cell division. For example, in the knockout mutant of *ABERRANT LATERAL ROOT FORMATION 4* (*ALF4*), patches of pericycle cells expressing *DR5:GFP* are specified as founder cells, but do not develop as LRPs due to a defect in cell cycle progression (DiDonato et al., 2004; Dubrovsky et al., 2008). This is might be attributed to the inhibition of auxin signaling, as the SCF^{TIR1} substrate IAA7 is stabilized in *alf4* mutants (Bagchi et al., 2018). Two auxin responsive genes, *MEMBRANE ASSOCIATED KINASE REGULATOR 4* (*MAKR4*) and *LATERAL ORGAN BOUNDARIES-DOMAIN 16/ASYMMETRIC LEAVES2-LIKE 18* (*LBD16*/*ASL18*), are also known to mediate the transition of the founder cell into a LRP (Goh et al., 2012; Xuan et al., 2015). Both *MAKR4* and *LBD16* are expressed in the founder cells before nuclear migration, and are necessary for the activation of the founder cells to undergo asymmetric cell division.

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Artificially decreasing *MAKR4* transcription or interrupting LBD16-dependent transcription do not affect the founder cell specification, but result in a reduced number of LRPs and LRs because of arrested founder cells. However, the underlying mechanism these genes are acting through remains elusive.

Root clock response to environmental cues

Essential for the high level of plasticity that is typical of roots, LR development is largely influenced by environmental factors (Lavenus et al., 2013; Motte et al., 2019). Previous results have shown that pre-branch sites are produced at a similar rate under altered growth conditions (e.g. different temperatures, photoperiod and nutrient supply) regardless of the changes these conditions have on primary root elongation rate, implying an independent and inherent 'ticking' of the root clock (Moreno-Risueno et al., 2010). However, recent studies have suggested that different environmental stimuli elicit diverse responses from the root clock.

For example, light is required for maintaining the oscillating signal and pre-branch site formation (Kircher and Schopfer, 2018). The absence of light results in a strong suppression of both primary root elongation and pre-branch site formation, which can be reversed by supplemental illumination in a dose-dependent manner. This light-mediated regulation of pre-branch site formation is achieved by the activation of the tryptophan-dependent auxin biosynthesis pathway. Meanwhile, an increase of non-persistent pre-branch sites is also observed in dark conditions (Kircher and Schopfer, 2018). These pre-branch sites no longer express *DR5:Luciferase* and fail to develop into LRPs.

Water availability is also known to regulate periodic root branching. When roots completely lose contact with water, such as in air spaces in the soil, endogenous abscisic acid accumulates in the root, which in turn disrupts the oscillations and pre-branch site formation (Orman-Ligeza et al., 2018). Conversely, uneven distribution of water in space restricts the sidedness of LRs without affecting oscillation and pre-branch site formation. The pre-branch sites oriented towards the water-contact side are specified and activated for further development, whereas pre-branch sites in the water-free side are arrested at the founder cell stage (Bao et al., 2014).

Plants can bypass the root clock to form lateral roots

The root clock acts as an endogenous cue to pattern LRs along the primary root with a regular spacing. However, under specific conditions (discussed below), abnormal LR formation independent of the root clock can be observed. This suggests plants can also achieve further branching plasticity through bypassing the root clock.

For example, applying exogenous auxin is able to promote *de novo* formation of extra pre-branch sites in the differentiation zone independent of the root clock, whereas treatment with the auxin transport inhibitor prenoylbenzoic acid induces extra LRs to be formed opposite one another (Kircher and Schopfer, 2016, 2018). Furthermore, in NPA-pretreated primary roots, which lack oscillation and pre-branch sites (Xuan et al., 2016), 1-aphthylacetic acid (NAA) treatment induces auxin signaling in the XPP cell layer and activates local cell division (Himanen et al., 2002). The activation of cell division by NAA is synchronized in the entire XPP cell layer, and produces longitudinal (along a xylem pole) and radial (along opposite xylem poles) clusters of LRPs along the primary root, instead of a regularly spaced pattern dictated by the root clock.

A recent study has reported that the rootless mutant *arf7arf19*, which is defective in LRI (Okushima et al., 2007), regains the capacity to produce LR-like organs upon wounding and drought stress or in soil conditions (Sheng et al., 2017). These peculiar lateral organs exhibit a clustered distribution along the primary root and are not generated by oscillations, because their formation is not affected when the root tip (including the OZ) is decapitated. Instead, the formation of these lateral organs in *arf7arf19* mutants requires shoot-derived auxin, and is mediated by the transcription of WUSCHEL-related homeobox gene *WOX11*. WOX11 regulates adventitious root initiation in the shoot and hypocotyl, but does not participate in LRI. Thus, the WOX11-mediated root branching in *arf7arf19* mutants under stress conditions, suggests the presence of an uncharacterized mechanism, which functions independently of the root clock.

Conclusions and perspectives

Periodic LR branching along the primary root axis provides a perfect model with which to investigate the fundamental processes that encode and translate spatial and temporal information into a developmental signal in plants. The pre-patterning processes that determine lateral organ spacing along the main root are complex: they involve the integration of a recurrent signal (oscillation) into a persistent development signal (pre-branch site formation), and the activation of the pre-branch site into a lateral organ. Several molecular and genetic signaling components have been shown to act as temporal or positional cues that regulate pre-patterning processes. Although these components have been studied separately through independent studies, the way they are coordinated remains unclear. Moreover, it is obvious that critical cues involved in pre-patterning processes are still undiscovered.

As we have discussed, the plant hormone auxin is crucial for LR development and has a major effect on the amplitude of the oscillations through its tissue-specific biosynthesis, directional transport and local signaling transduction. Thus, auxin is required – but is not sufficient – to trigger the oscillations. The oscillation amplitude reflects the activation of ARFs resulting from the degradation of AUX/IAA proteins by a local auxin input. However, several LR-associated ARFs, AUX/IAAs and auxin flux carriers are not – or are only partially – involved in the generation of the oscillations. These results indicate functional redundancy between these proteins, or the involvement of uncharacterized ARFs or auxin transporters. Furthermore, there is increasing evidence for the involvement of cell-to-cell communication systems in the generation of the oscillation and specification of the founder cells for LR formation. Signals derived from the LRC (e.g. auxin) and the differentiation zone (e.g. carotenoid derivatives) outside the OZ are found to regulate the oscillations in the OZ, whereas a local lateral inhibition mechanism prevents several LRs from developing within the same pre-branch site. These findings highlight the involvement of a non-cell-autonomous regulation mechanism during the pre-patterning process, which could be mediated by mobile signals such as auxin, cytokinins, peptides and carotenoid derivatives. Further investigations are necessary to address how these mobile signals influence the root clock, and how these cues are transduced towards a local signal. This will require the use of complementary approaches, such as forward genetics and computational modeling (Laskowski and ten Tusscher, 2017). Indeed, the latter has been recently shown to be useful to generate alternative hypotheses for the establishment of the oscillation pattern (Box 2) (van den Berg and ten Tusscher, 2018 preprint).

In addition to the endogenous signaling network, environmental factors are able to influence lateral root formation but at different

developmental steps. A recent study suggests that excessive exposure to the heavy metal cadmium inhibits pre-branch site formation through the repression of periodic LRC-PCD and *DR5* oscillations (Xie et al., 2019). In *Arabidopsis*, heterogeneous distribution of nutrients in the soil, such as phosphate and nitrogen, also largely influence the primary root and LR development through moderating auxin signaling, transport and cell cycle progression (Guan et al., 2017; Lima et al., 2010; O'Brien et al., 2016; Perez-Torres et al., 2008). However, it remains unclear whether the oscillation and pre-branch site formation are influenced by nutrient availabilities. Interestingly, *LOW PHOSPHATE ROOT1* (*LPR1*) and *NITRATE TRANSPORTER 1* (*NRT1*) are expressed in the root cap (Krouk et al., 2010; Svistoonoff et al., 2007), which supports the idea of the root cap being involved in mediating nutrient-adapted LR patterning (Crombez et al., 2019). Future research should, however, focus on the effect of nutrients on root pre-patterning.

In the model plant *Arabidopsis*, the primary root shows a bilateral symmetric (i.e. diarch) vascular bundle consisting of two poles of xylem elements and two poles of phloem elements. Interestingly, LRs develop along the primary root in a left-right alternating pattern, although both xylem poles seem to be subject to a single oscillation event. During each oscillation, XPP cells situated at only one of the two xylem poles become specified as LR founder cells which raises the question of how and when the LR 'sidedness' is determined. The lack of cellular resolution in observations made with the *DR5:Luciferase* reporter, impedes a detailed view of what is happening at the cellular level during the oscillations and pre-branch site establishment. Detailed high resolution *in vivo* imaging using appropriate reporter genes might help to resolve this longstanding enigma.

Finally, a regular-spaced pattern of LRs has been observed in other plant species with a tap root system (Barlow and Adam, 1988; Chen et al., 2018). Thus, it would be interesting to investigate whether this rhythmic pattern of LR formation correlating with oscillation in gene expression is representing a conserved mechanism in tap root systems or even in the fibrous root systems of monocotyledonous plants. The application of the auxin-responsive *DR5* promoter fused with appropriate reporters is an excellent tool for studying auxin response and LR events in a diverse range plant species (Chen et al., 2018; Rellán-Álvarez et al., 2015), and will help to confirm the action of the root clock in other plant species.

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Competing interests
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